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Splicing Inhibition at the Level of Spliceosome Assembly in the Presence of Herpes Simplex Virus Protein ICP27

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Herpes simplex virus (HSV) immediate-early protein ICP27 is a multifunctional regulator of viral and cellular gene expression. It has previously been shown that ICP27 directly or indirectly modulates several posttranscriptional processes, such as pre-mRNA splicing and polyadenylation. We show here that pre-mRNA splicing is inhibited in nuclear extracts prepared from cells in which ICP27 has been transiently expressed. Our results show that splicing inhibition in ICP27 extracts is manifested at early stages of the splicing process. Furthermore, our results suggest that an enzymatic activity in ICP27-containing extracts causes the splicing inhibition.

Key Words: ICP27; herpes simplex virus; mRNA; splicing.

INTRODUCTION

Herpes simplex virus (HSV) is a nuclear replicating DNA virus that encodes approximately 80 proteins. The HSV genes are expressed in a temporal cascade in which the immediate-early (IE) genes are expressed first, followed by the expression of the early (E) and late (L) genes. Lytic HSV infection involves a series of transcriptional and posttranscriptional processes, resulting in a sequential activation and repression of IE, E, and L genes and in an almost complete shutoff of host cell gene expression. Contrary to cellular protein coding genes only 4 of approximately 80 HSV genes contain introns (reviewed in Roizman and Sears (1996)).

ICP27 is an IE protein that is essential for productive HSV infection in cultured cells (Sacks *et al.,* 1985). HSV mutants with defective or absent ICP27 proteins express reduced levels of some E and most L mRNAs and are defective in viral DNA replication (McCarthy *et al.,* 1989; McMahan and Schaffer, 1990; Rice and Knipe, 1990). During a lytic HSV infection host cell gene expression is drastically reduced and a considerable amount of data has suggested that ICP27 causes the shutoff of cellular gene expression by inhibiting pre-mRNA splicing: cellular gene expression is not suppressed in the presence of mutant ICP27 (Hardwicke and Sandri-Goldin, 1994; Phelan *et al.,* 1993). Since the majority of HSV genes do not contain introns, HSV gene expression would be relatively resistant to the splicing inhibition. The mechanism(s) by which ICP27 carries out its different activities is largely unknown. However, a large number of studies have demonstrated that at least some, if not most, of its effects on gene expression occur at the posttranscriptional level. For instance, studies have demonstrated that ICP27 can modulate the pre-mRNA polyadenylation efficiency (McGregor *et al.,* 1996; McLauchlan *et al.,* 1992), promote nucleocytoplasmic export of intronless HSV genes (Sandri-Goldin, 1998a), increase the stability of labile mRNAs (Brown *et al.,* 1995), and change the intranuclear distribution of pre-mRNA splicing factors (Phelan *et al.,* 1993; Sandri-Goldin *et al.,* 1995) and it has also been proposed to inhibit pre-mRNA splicing (Hardy and Sandri-Goldin, 1994; Sandri-Goldin and Mendoza, 1992). Also, ICP27 has been reported to coimmunoprecipitate with splicing factors (Sandri-Goldin, 1998b; Sandri-Goldin and Hibbard, 1996) and to alter the phosphorylation status of some of these proteins (Sandri-Goldin and Hibbard, 1996). Finally, ICP27 has been shown to have RNA-binding activity and to continuously shuttle between the nucleus and the cytoplasm (Ingram *et al.,* 1996; Phelan and Clements, 1997; Sandri-Goldin, 1998a; Soliman *et al.,* 1997).

We here present a cell-free *in vitro* splicing system that allows studies of ICP27-caused splicing inhibition. The system is based on splicing of exogenous pre-mRNAs in nuclear extracts which are prepared from cells in which ICP27 has been transiently expressed. Results from our studies using this system show that pre-mRNA splicing activity is drastically reduced in nuclear extracts prepared from ICP27-expressing cells compared to control extracts. The specificity of the ICP27-caused splicing inhibition was supported by the fact that RNA polymerase II activities in nuclear extracts from both ICP27-containing and control cells were comparable. We found that the ICP27-caused splicing inhibition could be re-

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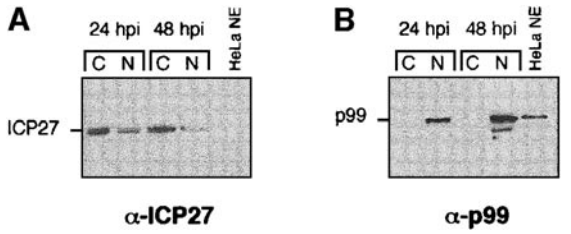

FIG. 1. ICP27 expression from recombinant adenovirus Ad5-ICP27. HeLa cells were either infected with Ad5-ICP27 or mock-infected, and ICP27 expression was induced for 24 or 48 h. Cells were harvested and fractionated into cytoplasmic (C, lanes 1 and 3) and nuclear (N, lanes 2, 4, and 5) extracts. Proteins were analyzed by SDS–PAGE and immunoblotting with either α-ICP27 antibodies (A) or α-p99 antibodies (B), using an ECL detection assay.

versed by addition of nuclear extracts from control cells, suggesting that only one or a few essential splicing factors are repressed. Furthermore, we find that the splicing inhibition is manifested at early stages of spliceosome formation, which suggests that splicing factors involved in the initial stages of the process are hampered in the presence of ICP27. Finally, we present data showing that the ICP27-mediated splicing inhibition can, at least partly, be transferred to splicing-competent nuclear extracts upon preincubation at 30°C, indicating that ICP27-containing extracts are associated with an enzymatic activity that results in splicing inhibition.

RESULTS

Expression of ICP27 in HeLa cells using recombinant adenovirus vector

In order to establish a reproducible method to transiently express HSV ICP27 protein in cell cultures we constructed a recombinant adenovirus encoding the protein. The adenovirus E1A and E1B genes were replaced with ICP27-coding sequences under tight control of a CMV progesterone promoter and a lac-repressor element (see Materials and Methods for details). The resulting virus, Ad5-ICP27, cannot replicate in HeLa cells since the viral proteins from the E1A and E1B regions are essential for efficient expression of other viral genes and for viral DNA replication. HeLa cells were infected with the Ad5-ICP27 virus and an activator virus encoding a chimeric transactivator protein containing the ligand-binding domain of hPRB891 fused to the Gal4 DNA-binding domain and the HSV VP16 transactivator domain (Molin *et al.,* 1998). At the onset of infection, ICP27 expression was specifically induced by addition of RU486, a progesterone antagonist. After 24 or 48 h of RU486 induction, HeLa cells were harvested and soluble cytoplasmic and nuclear protein extracts were prepared. We next analyzed the presence of ICP27 using Western blot assay with an ICP27-specific antibody. As shown in Fig. 1A, at 24 h after RU486 induction ICP27 is detected in both the cytoplasmic and the nuclear fractions of HeLa cells. An additional 24 h of induction did not markedly increase ICP27 levels (FIg. 1A, 48 hpi). As expected, no ICP27 could be detected in mock-infected cells (Fig. 1A, HeLa NE), demonstrating the specificity of the Western blot assay. Notably, the majority of soluble ICP27 was recovered in the cytoplasmic fraction (Fig. 1A, compare lanes C and N). Numerous previously published studies on ICP27 have indicated that at steady state it is predominantly a nuclear protein (Ackermann *et al.,* 1984; Knipe *et al.,* 1987; Mears and Rice, 1998; Phelan *et al.,* 1993; Sandri-Goldin, 1998b; Soliman *et al.,* 1997). One possible explanation for the discrepancy between our results and previously published observations was that our cytoplasmic extracts became contaminated with nuclear proteins during cell fractionation. To test this hypothesis we analyzed the distribution of p99 in our cell extracts. p99 is an almost exclusively nuclear protein in interphase cells and its presence in the cytoplasmic fraction would therefore be an indicator of nuclear contamination (Kreivi *et al.,* 1997). The nitrocellulose filter that originally was probed with ICP27 antibodies (Fig. 1A) was stripped and reprobed with p99-specific antibodies. As seen in Fig. 1B, the vast majority of p99 was detected in the nuclear fractions, with no detectable p99 in cytoplasmic extracts prepared from 24-hpi cells and only low levels of p99 in cytoplasmic extracts from cells that had been induced for 48 h (Fig. 1B). Thus, the presence of ICP27 in the cytoplasmic fraction is probably not the result of nuclear contamination of the cytoplasmic extract. Possible explanations for the discrepancy between our results and the majority of previously published results regarding ICP27's cellular distribution are presented elsewhere (see Discussion). Collectively these results show that ICP27 is readily expressed from Ad5-ICP27 in HeLa cells and under the conditions in which the cytoplasmic and nuclear extracts were prepared, most of the soluble pool of ICP27 was present in the cytoplasmic fraction (see Discussion for more details on ICP27 distribution and solubility).

Splicing is inhibited in nuclear extracts prepared from ICP27-expressing cells

To determine the effect of ICP27 on the pre-mRNA splicing process, we compared splicing activity in ICP27-containing nuclear extracts with the activity in extracts prepared from mock-infected cells. Two different ^{32}P-labeled pre-mRNA substrates, a β-globin pre-mRNA and an adenovirus derived pre-mRNA, were incubated under *in vitro* splicing conditions in the two types of extracts. As shown in Fig. 2A, β-globin and adenovirus pre-mRNAs were efficiently spliced in nuclear extracts prepared from mock-infected HeLa cells (lanes 1 and 4). The nuclear extracts prepared from ICP27-expressing cells, on the other hand, possessed low, if any, splicing activity (Fig.

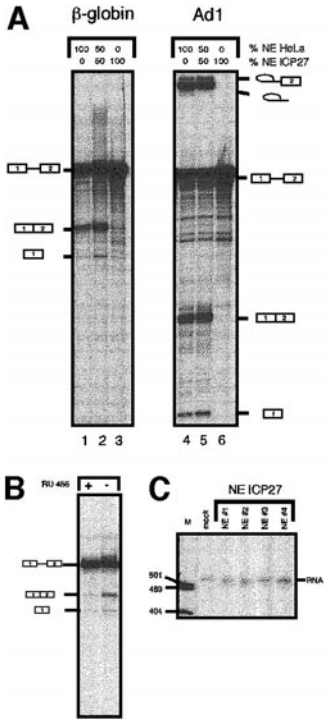

FIG. 2. Comparison of *in vitro* splicing and RNA polymerase II activities in ICP27-containing extracts and HeLa mock extracts. (A) *In vitro* splicing reactions using ^{32}P-labeled Ad1 and β-globin pre-mRNA as substrates were performed for 1 h in nuclear extracts prepared from mock-infected HeLa cells and from ICP27-expressing HeLa cells. Indicated amounts of ICP27-containing nuclear extracts and control extracts were used in the splicing reactions. The position of pre-mRNA and the various splice products are indicated on the sides. (B) *In vitro* splicing of a β-globin pre-mRNA substrate in nuclear extracts prepared from HeLa cells infected with the ICP27 expressing recombinant adenovirus Ad5-ICP27. ICP27 expression was either induced (+) with RU486 or not (−). (C) RNA polymerase II reactions were performed in nuclear extracts under optimal conditions in the presence of α-^{32}P-labeled CTP and a DNA fragment containing the adenovirus major late promoter. The expected size of the RNA polymerase II product is indicated on the right.

2A, lanes 3 and 6). The input pre-mRNAs were stable in ICP27 extracts during the course of the experiments, arguing that the decreased levels of splicing products and intermediates are not likely to be the result of increased RNA degradation. In order to test whether splicing could be initiated in the presence of ICP27, the splicing-competent control extracts were mixed with ICP27-containing extracts at a 1:1 ratio and immediately analyzed for splicing activity. As shown in Fig. 2A, the addition of splicing-competent extracts to the ICP27-containing nuclear extracts completely restored splicing (Fig. 2A, compare lanes 2 and 3 and lanes 5 and 6). The β-globin and adenovirus pre-mRNAs were at least as efficiently spliced in mixed extracts as in control extracts: a quantification of the gels presented in Fig. 2A demonstrated that the β-globin pre-mRNA was 10% more efficiently spliced in the mixed extracts than in the control extracts and that the adenovirus pre-mRNA was equally well spliced in mixed and control extracts. In identical mixing experiments we have noted that splicing is notably activated in several batches of ICP27-containing nuclear extracts when complemented with control extracts at a 1:3 ratio (data not shown), suggesting that the overall quality of the ICP27 extracts is good and that only a few components of the splicing machinery are targeted by ICP27 (discussed in more detail under Discussion). The experiments presented in Fig. 2A have been repeated several times using different preparation of ICP27 extracts and, although there is some variability in the degree of inhibition, we have consistently observed an 80–100% reduction in splicing activity in ICP27 extracts compared to the control extract, which was used in all experiments presented here except Fig. 2B.

In order to confirm that it was expression of ICP27 that causes the splicing observation presented in Fig. 2A, we prepared nuclear extracts from uninduced or RU486-induced HeLa cells that had been infected with Ad5-ICP27. The cells were otherwise treated identically and the nuclear extracts were prepared in parallel. An immunoblotting analysis of the resulting extracts confirmed that only low levels of ICP27 were expressed in the uninduced cells, whereas ICP27 was abundant in the extracts from RU486-induced cells (data not shown). We next compared splicing activity in the nuclear extracts from RU486-induced cells or uninduced cells. As shown in Fig. 2B, RU486 induction (and thus ICP27 expression) caused a dramatic reduction of splicing activity in the resulting extracts. Although the splicing activity in the extracts from the uninduced cells was relatively low compared to the HeLa extracts used in Fig. 2A (lanes 1 and 4), it was substantially higher than that in the ICP27-containing extracts that were prepared in parallel (compare lanes + and − in Fig. 2B). Moreover, we did not observe any dramatic reduction in splicing activity in nuclear extracts prepared from HeLa cells expressing chloramphenicol acetyltransferase from an identical recombinant adenovirus vector (data not shown). From these results we conclude that the negative effect of ICP27 on splicing is not due to toxicity from the virus infection as such or to protein overexpression.

We next sought to test whether the reduced splicing activity observed in ICP27 extracts was specific for the splicing process. For this purpose we analyzed the activity of another nuclear process in ICP27 extracts and control extracts: RNA polymerase II transcription. A DNA fragment containing the adenovirus major late promoter was incubated together with α-^{32}P-labeled CTP in ICP27 extracts or control extracts under conditions optimal for RNA polymerase II transcription. We analyzed RNA polymerase II activity in different preparations of nuclear extracts from ICP27-expressing cells and mock-infected cells. As can be seen in Fig. 2C, no significant differences in RNA polymerase II activity in the different preparations of extracts were detected. Importantly, RNA

polymerase II activity in ICP27-containing extracts was not lower than that in extracts from mock-infected cells. Taken together, the RNA polymerase II assay confirms that the splicing inhibition observed in ICP27 extracts is specific, rather than a general toxic effect of an overexpressed protein.

ICP27 inhibits splicing at early stages of spliceosome assembly

We next sought to determine at which stage of the splicing process the negative effect of ICP27 is first manifested. Mechanistically, pre-mRNA splicing is a two-step reaction which is catalyzed in a ribonucleoprotein complex called the spliceosome (reviewed in Burge *et al.* (1998)). Besides pre-mRNA, spliceosomes contain U snRNP particles and non-snRNP splicing factors. *In vitro,* spliceosomes are formed in a stepwise manner where the E complex is the first discrete functional spliceosomal complex. It contains U1 snRNP and several other non-snRNP splicing factors, which associate with the pre-mRNA in an energy-independent manner. The E complex is converted into the pre-spliceosomal A complex after ATP hydrolysis and addition of U2 snRNP. Following additional ATP hydrolysis, U4/U6.U5 snRNP is added to A complex and thereby converts it into the prespliceosome B complex. After further ATP hydrolysis and rearrangements within the B complex, the C complex is formed, which is the mature spliceosome in which the two catalytic steps of splicing are catalyzed. The assembly of pre-mRNA into spliceosomal complexes A, B, and C, but not E complex, can be studied *in vitro* by separating splicing reactions using native polyacrylamide gel electrophoresis. The A, B, and C complexes have different kinetics and they separate into three relatively distinct units during electrophoresis (Fig. 3A). The E complex, however, is "hidden" within the nonspecific H complex and can therefore not be distinguished using this assay. The exact nature of the H complex is not clear. Besides the E complex, the H complex also contains RNA–protein complexes that are not functional spliceosome precursors. In an attempt to identify the mechanism by which splicing is inhibited in ICP27 extracts we compared spliceosome assembly kinetics in ICP27 extracts with that in control extracts. A ^{32}P-labeled pre-mRNA was incubated under *in vitro* splicing conditions in ICP27 extracts and control extracts, and at various time points the splicing reactions were stopped and the samples were divided into two halves. One half was analyzed for spliceosome formation and from the other half RNA was isolated and analyzed for splicing products. As shown in Fig. 3A, splicing complexes A, B, and C are readily formed in control extracts from mock-infected HeLa cells. The splicing catalytic C complex was already detected in control extracts after 15 min of incubation (Fig. 3A, lane 2). In agreement with this, RNA products

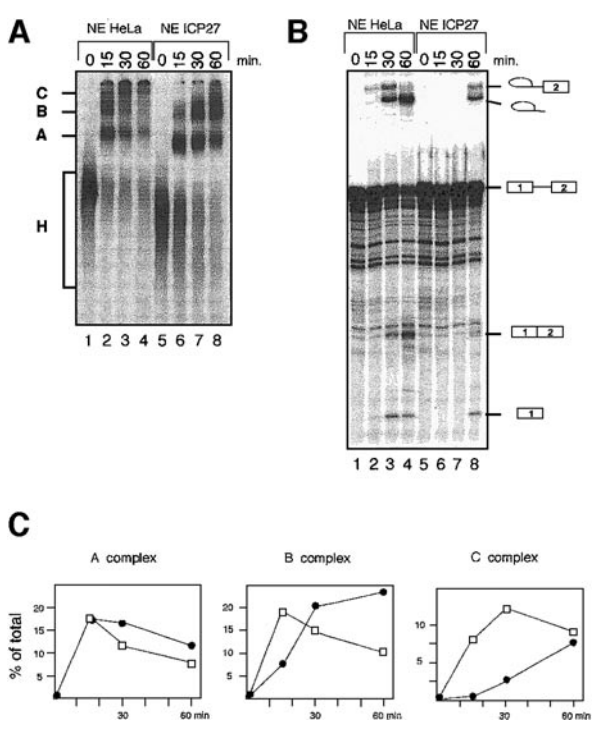

FIG. 3. Analysis of splicing complex formation in ICP27-containing extracts. *In vitro* splicing reactions were performed in ICP27-containing nuclear extracts and control extracts using ^{32}P-labeled Ad1 pre-mRNA as substrate. At indicated time points samples were withdrawn and directly analyzed for spliceosome formation using native polyacrylamide gel electrophoresis (A), or RNA was isolated and production of splicing products was analyzed using denaturing polyacrylamide gel electrophoresis (B). The positions of the different splicing complexes are indicated on left-hand side of A and splicing products are indicated on the right-hand side of B. (C) The quantitative results of the relative accumulation of the spliceosomal complexes A, B, and C in panel (A). The relative accumulation is expressed as the percentage of total RNA in each lane. Filled circles represent complexes formed in ICP27 extracts and hollow boxes represent complexes formed in control extracts.

from the first catalytic splicing step were observed at this time point (Fig. 3B, lane 2). It is also noteworthy that the amounts of prespliceosomal A and B complexes in control extracts started to decline between 15 and 30 min of incubation, whereas the amount of C complex continued to accumulate for up to 30 min of incubation (Fig. 3A, lanes 1–4; a quantitative analysis of the gel is presented in Fig. 3C). In ICP27-containing extracts, however, quite a different picture emerged. Although the amount of A complex was as abundant in ICP27 extracts as in control extracts after 15 min of incubation, substantially less B complex and hardly any C complex had formed in ICP27 extracts at this time point (Fig. 3A, lanes 2 and 6 and filled circles in Fig. 3C). After 30 min of incubation in ICP27 extracts the level of B complex was higher than in control extracts (Fig. 3C). The B complex continued to accumulate in ICP27 extracts through the course of incubation, whereas in control extracts the amount of B complex peaked around 30 min. A probable explanation for the differences in kinetics is that B complex is more

efficiently converted into C complex in control extracts as compared to ICP27 extracts. In support of this we note that the largest differences in formation of spliceosomal complexes between ICP27 extracts and control extracts appears to be in the rate at which C complexes are formed (Fig. 3C). As expected, the slower formation of C complex in ICP27 extracts was accompanied by reduced accumulation of splicing products (Fig. 3B, compare lanes 2 to 4 with lanes 6 to 8). Splicing products are readily observed after 15 min of incubation in control extracts, but it takes more than 30 min of incubation in ICP27 extracts in order for detectable amounts of splicing intermediates to be produced (Fig. 3B, lane 8). In separate experiments, using incubation times shorter than 15 min, we have noticed that the rate of A complex formation is also slower in ICP27 extracts than in control extracts (data not shown), thus demonstrating an effect by ICP27 on early stages of spliceosome assembly. In addition to the differences in the rates at which the different spliceosomal complexes are formed, we note that the A complex formed in ICP27 extracts consistently migrates notably faster than A complex formed in control extracts (Fig. 3A, compare lanes 2 to 4 with lanes 6 to 8). Also, although it is not as obvious as for the A complex, there is a clear tendency for H and B complexes formed in ICP27 extracts to migrate faster than their counterparts formed in control extracts. The faster mobility of the RNA–protein complexes formed in ICP27 extracts could reflect a qualitative and/or a quantitative difference between these and their counterparts formed in control extracts. We note that the protein concentrations in the various ICP27 extracts and control extracts are comparable (data not shown). It is therefore unlikely that the faster mobility of RNA–protein complexes formed in ICP27 extracts result from lower concentrations of splicing factors and other RNA-binding proteins. We are currently investigating whether the different mobilities of spliceosomal complexes formed in ICP27 extracts and control extracts reflect a qualitative difference between prespliceosomes and spliceosomes. In summary, the results presented in Fig. 3 combined with results from a similar experiment show that the reduced splicing rate in ICP27 extracts is manifested already at the formation of the first energy-dependent prespliceosome complexes (A and B complexes). Moreover, they show that the rates in which A complex is converted to B, B complex to C complex, and C complex to splicing products are all reduced in ICP27 extracts. However, the largest difference between ICP27 extracts and control extracts appears to be the rate at which C complexes, i.e., mature spliceosomes, are formed.

ICP27's splicing inhibitory activity can be transferred to splicing-competent nuclear extracts

It has previously been reported that splicing activity in nuclear extracts prepared from HSV-infected HeLa cells is reduced compared to splicing in extracts isolated from cells infected with an ICP27 viral mutant (Hardy and Sandri-Goldin, 1994). Furthermore, in the same report it was shown in biochemical complementation assays that HSV-caused splicing inhibition could be transferred to otherwise splicing-competent extracts in a dominant way. Contrary to this, we found that a 1:1 mixture of ICP27 extracts and control extracts was sufficient to fully restore splicing (Fig. 2A, lanes 2 and 4). In these experiments the extracts were mixed on ice and immediately used in splicing assays. Thus, it was possible that a putative *trans*-dominant effect of ICP27 did not have time to act on the splicing machinery in control extracts before the splicing process had initiated. We therefore redesigned the complementation experiments in order to test whether the splicing inhibitory activity in ICP27 extracts could be transferred to otherwise splicing-competent extracts. The splicing-competent control extracts were mixed with ICP27 extracts at a 1:1 ratio and incubated at 30°C for various times or on ice for 2 h. At the end of the preincubation, splicing was initiated by addition of ^{32}P-labeled pre-mRNA, magnesium, creatine phosphate, and ATP. As shown in Fig. 4, the amount of spliced mRNA was reduced in mixed extracts that had been preincubated for 1 h at 30°C (compare lanes 1 and 3). Also, the negative effect of ICP27 extracts on control extracts was enhanced with time of preincubation at 30°C (Fig. 4, compare lane 1 with lanes 3–7). Splicing was substantially less reduced in mixed extracts that had been preincubated on ice (Fig. 4, lane 8). Splicing activity in nuclear extracts from mock-infected cells was also reduced upon preincubation at 30°C (Fig. 4, compare lanes 1 and 2), but the reduction was not as large as in mixed extracts. Note that although the lariat splicing intermediate intron–exon 2 and the spliced-out intron did not separate well in the experiment presented in Fig. 4, quantitative analyses of this and similar experiments demonstrate that splicing is inhibited before the first catalytic step also in the preincubated mixed extracts (data not shown). These results taken together indicate that a dominant splicing inhibitory activity is present in ICP27 extracts and that this activity requires time and 30°C to accomplish the inhibition.

DISCUSSION

In this study we present an assay in which ICP27-caused splicing inhibition for the first time is studied *in vitro* in the absence of other HSV proteins. A large body of evidence has supported the notion that ICP27 inhibits splicing (Hardwicke and Sandri-Goldin, 1994; Hardy and Sandri-Goldin, 1994; Sandri-Goldin and Mendoza, 1992; Soliman *et al.,* 1997). For instance, nuclear extracts from HSV-infected HeLa cells have lower splicing activity than extracts isolated from cells infected with ICP27 null mutants (Hardy and Sandri-Goldin, 1994), ICP27 coimmuno-

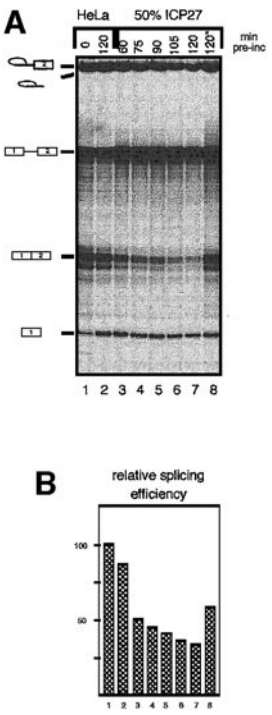

FIG. 4. Effect on *in vitro* splicing after mixing and preincubating ICP27-containing nuclear extracts with extracts from mock-infected HeLa cells. (A) ICP27 extracts were mixed with HeLa extracts (lanes 3 to 8). The samples in lanes 2 to 7 were incubated at 30°C for the indicated times (in minutes) and the sample in lane 8 was incubated on ice for 2 h. ^{32}P-labeled Ad1 pre-mRNA and ATP were added at the end of preincubation and splicing was allowed to proceed for 1 h before RNA was extracted and analyzed using denaturing polyacrylamide gel electrophoresis. The positions of the pre-mRNA and the various splice products are indicated on the right. Note that in this particular experiment the splicing intermediate *intron–lariat–exon 2* and the splicing product *intron–lariat* did not separate well during electrophoresis. (B) The relative splicing efficiencies in A were quantitated using a phosphorimager as described under Materials and Methods.

precipitates with various splicing factors (Sandri-Goldin, 1998b; Sandri-Goldin and Hibbard, 1996), and ICP27 redistributes splicing factors in nuclei of infected cells (Phelan *et al.,* 1993; Sandri-Goldin *et al.,* 1995). However, direct evidence for a splicing inhibitory function of ICP27 in the absence of other viral proteins has been lacking. In order to study the putative splicing inhibitory activity of ICP27, we constructed a eukaryotic expression system based on a recombinant adenovirus which would allow us to express relatively large amounts of the protein in HeLa spinner cells. We found that ICP27 was readily expressed in HeLa cells and although a substantial portion of ICP27 was recovered from nuclei, more of the protein was present in a soluble cytoplasmic cell fraction. In contrast to this finding, many previously published studies have shown that although ICP27 is continuously shuttling between the nucleus and the cytoplasm, the nuclear steady state levels of the protein are substantially higher than the cytoplasmic levels (Ackermann *et al.,* 1984; Knipe *et al.,* 1987; Mears and Rice, 1998; Phelan *et al.,* 1993; Sandri-Goldin, 1998b; Soliman *et al.,* 1997). The predominant nuclear localization of ICP27 has been observed both for ICP27 transiently expressed from plasmid DNA and for ICP27 expressed during lytic HSV infections. However, there is a precedence for cytoplasmic enrichment of ICP27 too: using a cell fractionation assay Wilcox *et al.* (1980) found that ICP27 is evenly distributed between the nucleus and the cytoplasm during a virus infection. In the same study it was demonstrated that the cytoplasmic pool of ICP27 is much more soluble in 0.15 M NaCl than ICP27 isolated from nuclei. They found that almost all ICP27 in the nuclear extracts precipitated upon dialysis from high-salt (0.5 M NaCl) to low-salt (0.15 M NaCl) buffer, whereas cytoplasmic ICP27 largely remained soluble upon an identical change in salt concentration. The nuclear and cytoplasmic extracts that we used in this study were prepared and treated in a similar way; the HeLa cell nuclei were extracted at 0.3 M KCl and the resulting extracts were dialyzed to 0.1 M KCl. It is thus likely that the majority of ICP27 originally present in our nuclear extract preparations precipitated during dialysis. From this follows that in the assay, which is presented in Fig. 1, we compare the soluble pool of ICP27 in the nucleus and cytoplasm. If a large portion of nuclear ICP27 actually is insoluble under the conditions used for nuclear extract preparation, the result would be an overestimation of cytoplasmic versus nuclear distribution of ICP27. In this context it is worth mentioning that we have compared the concentration of various splicing factors in nuclear extracts from ICP27-expressing cells with control extracts, without detecting any consistent or major quantitative differences with the two types of extracts (Fig. 1B; and data not shown).

In a second part of this study we analyzed the effect of ICP27 on splicing *in vitro.* Taken together, the data from these studies strongly suggest that the presence of ICP27, in the absence of other HSV proteins, causes a general inhibition of splicing. The fact that a small amount of splicing-competent control extracts were able to substantially activate splicing to ICP27 extracts suggests that an essential splicing factor that is limiting for the process is hampered in ICP27 extracts, rather than causing major damage to the entire splicing machinery.

How then is splicing inhibited in the presence of ICP27? Our results show that the formation of all energy-dependent spliceosome complexes was delayed in ICP27 extracts as compared to control extracts; the rates at which A complex is converted into B complex, and B complex to C complex, were notably slower in ICP27 extracts than in control extracts. It was also apparent that different RNA–protein complexes formed in ICP27 extracts migrated notably faster during electrophoresis than their counterparts formed in control extracts. In particular, H, A, and B complexes formed in ICP27 ex-

tracts migrate faster than those assembled in control extracts. It is very likely that the altered mobility reflects a qualitative and/or a quantitative difference between the splicing complexes formed in the two types of extracts; one or more splicing factors may be missing or present in smaller amounts in the splicing complexes formed in ICP27 extracts. Another possibility is that ICP27 causes modification of specific splicing factors and that this in turn could impair the splicing factor's ability to participate in spliceosome formation and/or destabilize its interaction with other splicing factors. In support of this hypothesis we found that ICP27 extracts indeed possessed a dominant splicing inhibitory activity. The dominant inhibitory activity in ICP27 could be transferred to splicing-competent control extracts only if the two extracts were preincubated at 30°C, and the inhibition increased with time (Fig. 4). These results suggest that an enzymatic activity in ICP27-containing extract targets an essential component(s) of the splicing machinery. In this context it is interesting to note that productive HSV infections have been reported to alter the phosphorylation state of some splicing factors (Sandri-Goldin and Hibbard, 1996). Perhaps more relevant, recently published results showed that ICP27 interacts with the β subunit of casein kinase 2 (CK2) in the yeast two-hybrid system, and CK2 activity coimmunoprecipitates with ICP27 from extracts prepared from HSV-infected cells (Wadd *et al.,* 1999). It is thus tempting to speculate that ICP27, via CK2, inactivates essential splicing factors by phosphorylating them at critical sites. Another possible mechanism by which splicing is inhibited in the presence of ICP27 may be that ICP27 physically binds to mRNA (both intron containing and intronless) and thereby prevent the mRNA from being spliced. In support of this hypothesis we have found that pre-mRNA co-immunoprecipitates from ICP27 extracts (data not shown). We believe that a *trans*-inhibiting model (i.e., where ICP27 inhibits splicing via an enzymatic activity) and a *cis*-model (where ICP27 inhibits by binding to pre-mRNAs) are not mutually exclusive.

Almost all previously published *in vivo* and *in vitro* studies of ICP27 have relied on comparison of mRNA accumulation in cells, or extracts from cells, infected with wild-type or ICP27 mutated HSV viruses. Since many E genes and all L HSV genes are dependent on ICP27 for their expression, it is difficult to determine what is a direct effect and what is an indirect effect by ICP27. For instance, the *in vitro* splicing inhibition observed in extracts from HSV-infected cells could have resulted from any viral protein that is dependent upon ICP27 for its expression. Also, it is well established that cells have antiviral defense systems, which sense an invading virus and react by shutting down several biosynthesis processes, such as translation. The possibility can therefore not be formally excluded that such shutdown processes may affect splicing as well. To the best of our knowledge this is the first time it has been proven *in vitro* that the presence of ICP27, in the absence of other HSV proteins, inhibits splicing.

Which splicing factor(s) then, is the target(s) for the splicing inhibition in ICP27-containing extracts? As previously mentioned, our *in vitro* splicing results indicate that an essential splicing factor which is limiting for the splicing process is affected in ICP27 extracts; we find that it is sufficient to add splicing-competent extracts to ICP27 extracts at a 1:3 ratio in order to substantially restore splicing. Furthermore, our results show that the ICP27-caused splicing inhibition is manifested at early stages of spliceosome assembly, as shown by a reduced rate of formation of the first ATP-dependent prespliceosome complex in ICP27 extracts. However, although the rate of spliceosome formation was reduced at this stage, the largest differences between ICP27 extracts and control extracts appeared to be in the rates at which the mature spliceosomes (C complexes) were assembled. This suggests that although the target(s) for ICP27-caused splicing inhibition is present already at early stages of spliceosome formation, its inability to function properly in the ICP27 context is most apparent in the transition from the prespliceosomal B complex to the mature spliceosome. Potential targets for ICP27-caused splicing inhibition were recently suggested by Soliman and Silverstein (2000). They found that the C-terminal part of ICP27 contains a motif which is present in Sm and Lsm (Sm-like) proteins. Both Sm and Lsm proteins are key parts of spliceosomal U snRNPs, which are essential components of the spliceosomes. In addition to their role in splicing, Lsm proteins have been found to play a role in mRNA degradation (reviewed in He and Parker (2000)). The putative Sm motif in ICP27 is located in the region which is required for suppression of splicing, and Soliman and Silverstein proposed that ICP27 requires its Sm motif to bind to the Sm complex and inactivate its function in splicing. This model would thus predict, as far as we understand, that ICP27 inhibits splicing by titrating out Sm proteins. We believe that nothing in the data presented in this study supports or contradicts Soliman and Silverstein's proposed model for ICP27-caused splicing inhibition.

We are currently developing a complementation assay based on fractionated HeLa cell nuclear extracts in order to identify splicing factors which are sufficient and essential to restore splicing activity in ICP27 extracts. Preliminary results from these studies show that a family of splicing factors, the SR-proteins, are not capable of reversing the splicing inhibition in ICP27 extracts (data not shown). The SR-proteins are the main targets for adenovirus-mediated inhibition of cellular pre-mRNA splicing (Kanopka *et al.,* 1998). It thus appears that, although HSV and adenovirus attack the same cellular process, i.e. splicing, they have evolved different systems, which are directed against different components of the splicing machinery. This makes sense since whereas adenovirus

is highly dependent on splicing of its pre-mRNAs (only 1 of approximately 40 different mRNAs is generated from an intronless gene), most HSV genes are intronless. Whereas adenovirus favors its own growth at the host cell's expense by modulating the splicing machinery to splice viral pre-mRNAs, HSV appears to attack an essential splicing factor(s) and thereby cause a complete shutoff of splicing. For this reason we believe that identification of the factor(s) that is targeted by ICP27 could be of general importance since it may target previously uncharacterized parts of the splicing machinery.

During preparation of this article, J. B. Clements and co-workers reported that ICP27 interacts with the essential splicing factor SAP145 during an HSV infection (see Bryant *et al.,* 2001). SAP145 is a component of the SF3b particle, which is implicated in tethering U2 snRNP binding to the branch site and is thus needed at early stages of spliceosome formation. Their findings thus support our observation that splicing is inhibited at early stages of spliceosome assembly.

MATERIALS AND METHODS

Cells and viruses

The ICP27-expressing recombinant adenovirus AdG5Trip(His)-ICP27 Lac (referred to as Ad5-ICP27 in text) was constructed as follows. A *Bst*YI fragment containing ICP27 coding sequences was inserted into the *Bam*HI site in transfer plasmid pAdG5Trip(His)-Lac Bam (Edholm *et al.,* 2001), generating the transfer plasmid pAdG5Trip(His)-ICP27 Lac. Three micrograms of AdG5Trip(His) ICP27 Lac was transfected into 293Lacl cells together with 1 μg DNA from adenovirus mutant dl309. The transfected cells were overlaid with DMEM/agar. Virus plaques were isolated and purified. Recombinant viruses were verified by restriction enzyme cleavage of Hirt extracted viral DNA (Hirt, 1967). One plaque containing the full-length ICP27 gene was selected and purified by a second round of plaque assay. High-titer stocks of AdG5Trip(His) ICP27 Lac were prepared essentially as described by Hitt *et al.* (1994) and the virus titer (FFU) was determined in 293 cells. The activator virus AdCMVProg has previously been described (Molin *et al.,* 1998).

Expression of ICP27 in HeLa spinner cells

HeLa spinner cells were grown in spinner medium containing 5% newborn calf serum to approximately 5×10^5 cells/ml before being harvested by centrifugation. The medium was removed and cells were suspended in 1/10 of the original culture volume and infected with transactivator virus AdCMV Prog and Ad2-ICP27. After a 1-h incubation at 37°C, the remainder of prewarmed spinner medium and calf serum was added, and ICP27 expression was induced by addition of RU486 (final concentration was 0.5 μM). After 24 or 48 h of induction, cells were harvested by centrifugation and nuclear extracts were prepared as previously described (Kreivi *et al.,* 1991) and the cytoplasmic fraction was used to prepare S100 extract as described in Dignam *et al.* (1983). Nuclear extracts from mock-infected cells and/or non-induced Ad5-ICP27-infected cells were prepared in parallel.

Immunoblotting

Proteins were separated by 10% SDS–PAGE and transferred to nitrocellulose membranes using a semidry transfer apparatus. The filter was blocked with TBS buffer (20 mM Tris, pH 7.5, 150 mM NaCl) containing 2% dry milk. Primary antibodies were monoclonal anti-ICP27 (ABI) or sheep polyclonal anti-p99 (Kreivi *et al.,* 1997). Secondary antibodies were either horseradish peroxidase-conjugated anti-mouse or anti-sheep antibodies. Between ICP27 and p99 probing, the filter was stripped with 100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris, pH 6.8, at 50°C for 30 min. Detection utilized a chemiluminescent substrate followed by exposure on Biomax MR film (Amersham Pharmacia Biotech).

In vitro splicing and *in vitro* transcription

The total splicing reaction volume was 20 μl and contained a ^{32}P-labeled Ad1 (Konarska and Sharp, 1987) or β-globin (Muhlemann *et al.,* 2000) pre-mRNA substrate (50,000 cpm/reaction), 1.5 mM $MgCl_2$, 40 mM KCl, 20 mM creatinephosphate, 2 mM ATP, 15 U RNasin, 12 mM HEPES, pH 7.9, and 40% (vol/vol) nuclear extract. RNA polymerase II transcription was done essentially as described in Martinez *et al.* (1994). Briefly, a *Sma*I fragment encompassing the adenovirus major late promoter was incubated together with nuclear extracts in the presence of ^{32}P-labeled CTP. The splicing reactions and the RNA polymerase II products were incubated at 30°C for 1 h if not otherwise stated, before RNA was isolated and analyzed on 10% polyacrylamide gel, followed by autoradiography. Dried gels were exposed to ^{32}P-sensitive phosphorimager screens and scanned on a phosphorimager from Bio-Rad (GS-250). Splicing efficiency and RNA polymerase II transcription were calculated by volume integration of appropriate RNA signals.

Analysis of spliceosome assembly

For analysis of spliceosome assembly, 20-μl *in vitro* splicing reactions, using ^{32}P-labeled Ad1 pre-mRNA as substrate, were incubated at 30°C. At the indicated time points, the *in vitro* splicing reactions were stopped by transfer of the tubes to ice. Ten microliters of the reactions was removed for analysis of splicing products as described above. Heparin was immediately added to the remaining half of the reactions and all of it was loaded on a 3.5% (60:1) native polyacrylamide gel. The gel and

running buffer were 75 mM Tris/glycine and the gels were run at 350 V for 4 h. Gels were dried and complexes were detected by autoradiography. The relative accumulations of various spliceosomal complexes were quantified by phosphorimager analysis on a Bio-Rad phosphorimager (GS-250).

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